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Microarray Analysis of Host and Pathogen Gene Expression Changes *In Vivo*

This application claims priority to United States Provisional Application No. 60/501,928, filed 10 September 2003.

Technical Field and Industrial Applicability of Invention

The present invention relates generally to system useful for investigating the dynamic global transcriptional or gene expression profiles of multiple distinct organisms from a local interaction, such as of a bacterial pathogen and host during the early phase of an *in vivo* infection.

Background of the Invention

The study of virulence and pathogenesis is of fundamental importance to understanding of the infectious disease process, as is the complementary study of the host immune response to the pathogen. Previously, transcriptional profiling has been used to characterize the global response of bacteria to a specific agent or mammalian cell, or the response of a cell line to a pathogen or pathogen components such as LPS, providing glimpses into the host/pathogen interaction. For example, studies examining the expression of intracellular *Salmonella enterica* in murine macrophage-like J774-A.1 cells utilized selective macrophage lysis after infection, furnishing insights into the bacterial response to the intravacuolar environment (Eriksson, S., *et al.*, *Mol Microbiol* 47, 103-118 (2003)). Similarly, several studies have identified host factors altered in response to a bacterial challenge using a variety of pathogens and cell lines (Rappuoli, R., *PNAS* 97, 13467-13469 (2000)).

The ability to examine the dynamic expression of both the host and the pathogen during the infection process have been severely hampered by several technological issues, such as the ability to obtain sufficient bacterial RNA for hybridization. For most bacterial microarray studies, the requirement of 1-20 micrograms of total RNA (for probe synthesis) necessitates a population of at least 10^9 cells (Bremer, H., et al., in *Escherichia coli and Samonella: Cellular and Molecular Biology*, F.C. Neidhardt, Ed. (ASM Press, Washington, D.C., 1996), Vol. 2, 1553-1569). A second problem is the difficulty in separating host and pathogen mRNA for

hybridization. Two previous studies used unique approaches to study host-free samples. *Vibrio cholerae* expression analysis isolated from rice water stool samples of cholera patients were compared to *in vitro* cultures, revealing several genes previously shown to be important for infection in infant mice (Merrell, D.S., *et al.*, *Nature* 417, 642-5 (2000)). In another study, the authors implanted a dialysis chamber in a rat peritoneum model in order to mimic a mammalian host-adapted state of *Borrelia burgdorferi* (Revel, A.T., *et al.*, *Proc Natl Acad Sci USA* 99, 1562-7 (2002)). A third technological problem has been the inability to amplify bacterial mRNA using standard oligo-dT T7 methodology employed for eukaryotic mRNA, due to the lack or paucity of poly (A) sequences on bacterial messages (Sarkar, N., in *Ann. Rev. Biochem.*, J. N. A. C. C. Richardson, C. R. H. Raetz, and J. W. Thorner, Ed. (Annual Reviews, Inc., Palo Alto, CA, 1997), Vol. 66, 173-197).

Summary of the Invention

The present invention provides, e.g., a method for examining changes in gene expression of multiple organisms coexisting in a sample comprising the steps of: extracting a sample of localized infection; amplifying RNA with and without *in vitro* RNA polyadenylation; labeling the RNA; and hybridizing the RNA to microarrays of the organisms. The invention also provides a method for analysis of gene expression of multiple organisms coexisting in a sample comprising the steps of: obtaining a sample from a local infection; extracting the RNA from the local infection; dividing the extracted RNA into at least two aliquots; polyadenylating the RNA in the first aliquot; carrying out reverse transcription of the RNA in the first aliquot and the second aliquot so as to synthesize a first strand of cDNA; synthesizing a second strand of the cDNA; amplifying the cDNA; labeling the resulting amplified product; hybridizing the resulting product from the first aliquot to a microarray containing bacterial genes and the product resulting from the second aliquot to a microarray containing host genes; and quantifying gene expression of the bacterial and host genes. The methods for analysis of gene expression of multiple organisms coexisting in a sample can be done from the same sample and in intervals over a time course of interaction. The present invention further provides a method for amplifying RNA

isolated from a mixed population of species comprising the steps of: obtaining a sample from a local infection; extracting the RNA from the local infection; dividing the extracted RNA into at least two aliquots; polyadenylating the RNA in the first aliquot; carrying out reverse transcription of the RNA in the two aliquots to synthesize a first strand of cDNA; synthesizing the second strand of the cDNA; and amplifying the cDNA. The local interaction in the various methods of the present invention can be, e.g., local infections infections, such as those based on an animal model (e.g., a murine granulomatous pouch model), or be samples taken from eukaryotes, preferably mammals, including humans. The present invention also provides a method for eliminating cross-hybridizing clones comprising the steps of: hybridizing probe samples from uninfected control animals to a bacterial microarray; hybridizing probe samples synthesized from RNA isolated from a bacterial culture to an animal microarray; and detecting hybridization of the samples. Other general aspects, specific embodiments, features, and advantages of the invention will be apparent from the following detailed description and the claims that follow.

Brief Description of the Figures

FIG. 1. Concordance correlation between pouch samples hybridized to the murine microarray.

FIG. 2. Concordance correlation between pouch samples hybridized to the *E. coli* microarray.

FIGS. 3 A & B. Concordance correlation between the medians of the time point sample groups. Correlation coefficients ranged from r=0.79-0.99 for the murine samples and r=0.46-0.97 for the bacterial samples. A) Murine microarray; B) *E. coli* microarray.

FIG. 4. Growth of *E. coli* C9 in murine pouch model. The standard error of the mean is shown for each time point, which included colony counts from 4-8 pouches.

FIGS. 5 A & B. Schematic representation of the host response to the *E. coli* pathogen, and protein levels observed in exudates from infected pouches from 0-24 hrs post infection. A) Fold increases in proteins involved in the acute phase response. B) Fold increases in proteins involved in the leukocyte recruitment. *, values are underestimates of fold change, due to exceeding Rules Based Medicine maximum value detected.

Detailed Description and Preferred Embodiments of the Invention

The present invention allows for the simultaneous transcriptional analysis from an *in vivo* infection of both the host and pathogen. This enhances the ability to amplify bacterial mRNA from the site of infection, and to distinguish host and pathogen (e.g., murine and *Escherichia coli*) expression without cellular separation. The invention provides a method for examining the changes in gene expression of both host and pathogen over time by modeling localized infection, amplifying RNA with and without *in vitro* RNA polyadenylation; and hybridizing the probes developed from the RNA to both pathogen and host microarrays. The present invention also provides a method for analysis of pathogen and host gene expression and a method for amplifying heterogeneous RNA.

The present invention advantageously facilitates the analysis and study of complex relationships between a host and pathogen, a long-standing goal for the field of infectious diseases. The combination of an animal model of localized infection with RNA amplification with and without *in vitro* RNA polyadenylation and subsequent hybridization of labeled cDNA probes to both host and pathogen (e.g. murine and *E. coli*) microarrays facilitates examination of global changes in gene expression of both the host and pathogen over time.

In one exemplary embodiment, a murine granulomatous pouch was adapted to serve as a model of localized infection and was investigated throughout the early phase of infection (24 hours). Pathogenic *E. coli* grew vigorously within the granuloma pouch, resulting in a host response to the infection and, in turn, a response by *E. coli* to

the host defenses. This combination of a localized animal infection model, amplification of both bacterial and host mRNA and subsequent transcriptional analyses using *E. coli* and mouse DNA microarrays made it possible to eavesdrop on the "infection conversation". The invention may be used for various studies of pathogenesis and of bacterial-host interactions, whether the interaction is pathogenic, commensal or symbiotic in nature.

Murine granuloma pouch model.

The dynamic changes in gene expression of both pathogen and host were examined in an *in vivo* infection model using a murine granulomatous pouch model (Dalhoff, A., *et al.*, *Infection* 10, 354-360 (1982)) innoculated with *E. coli* CP9, an extraintestinal pathogenic clinical isolate (ExPEC) which has been shown to be virulent in urinary tract, intraperitoneal and pneumonitis infection models (Russo, T. A., *et al.*, *Infection and Immunity* 69, 6209-6216 (2001)). To create a pouch, ~2 ml of air was injected intradermally between the shoulders of female Balb/c mice, followed by injection of 0.25 ml of 0.5% croton oil-99.5% olive oil into the space. After 5 days, 0.25 ml of a suspension of *E. coli* CP9 OD_{600} of 0.085 or 0.25 ml of PBS were injected into the pouches (time=0). The pouch fluids and blood were harvested immediately following euthanasia at time points from 0-24 hours post infection. Aliquots of each pouch were used for bacterial CFU determination as well as bacterial and murine RNA isolation. Analyte analysis of pooled pouch fluids from each time point was performed using Mouse MAP™ (Multi-Analyte Profiles) analysis technology (Rules Based Medicine, Inc, Austin, TX).

mRNA Amplification.

For bacterial mRNA amplification, poly A tails were appended to the 3' ends of total RNA isolated from the pouches using *E. coli* poly (A) polymerase (PAP) (Amersham Pharmacia Biotech, Piscataway, NJ). Bacterial total RNA amplified with T7 RNA polymerase and oligo dT-T7 primer following *E. coli* PAP treatment had a correlation coefficient of 0.95 when compared to total unamplified RNA in microarray experiments (data not shown). After cDNA synthesis using oligo dT-T7 primer and

PowerScript reverse transcriptase (Clontech, Palo Alto, CA), linear amplification of the purified products was performed using T7 RNA polymerase (Ambion, Austin, TX) and the AmpliScribe T7 Transcription Kit (Epicentre, Madison, WI) (see Examples for protocols). After purification, Cy3 coupled cDNA probe synthesis, microarray hybridization and quantitation were performed as previously described by Shaw, K. J., *et al., J. Mol. Microbiol. Biotechnol.* 5(2):105-22 (2003), as were eukaryotic RNA preparation, probe synthesis and microarray analysis (Simmen, K. A., *et al., Proc Natl Acad Sci USA* 98, 7140-5 (2001)).

Cross-hybridization.

Since RNA isolated from infected pouches contained both murine and *E. coli* mRNA, it was necessary to determine the extent of cross-hybridization to each microarray in order to identify and eliminate problematic genes from further analysis. To address this issue, *E. coli* CP9 total RNA was isolated from the initial inoculum and amplified without polyadenylation, using the standard protocol for murine RNA amplification, followed by Cy3 labeling and hybridization to the murine DNA microarray. This microarray contained a total of 3518 unique murine genes with some genes represented by multiple clones or spots. Of the 3518 unique genes, 59 were eliminated from further analysis due to cross-hybridization to *E. coli* RNA (see Examples). To address the issue of murine sequences cross-hybridizing to the bacterial microarray, RNA isolated from uninfected pouches was polyadenylated with *E. coli* PAP, amplified, Cy3-labeled, and hybridized to an *E. coli* MG1655 microarray (Shaw, K. J., *et al., J. Mol. Microbiol. Biotechnol.* 5(2):105-22 (2003)). Of the 4187 unique *E. coli* genes, 62 were eliminated from further analysis due to cross hybridization to murine sequences.

Correlation of gene expression patterns in mouse pouches.

The pouch fluids from a total of 55 mice were harvested, and both murine and bacterial mRNA were extracted and analyzed. These consisted of 43 infected pouches (0 hr, 8 pouches; 2 hr, 6 pouches; 4 hr, 7 pouches; 8 hr, 7 pouches; 12 hr, 6 pouches; 20 hr, 4 pouches; 24 hr, 5 pouches) as well as 12 non-infected pouches (3 pouches

each for 0 hr, 4 hr, 12 hr, 24 hr). Several samples were eliminated from the analysis due to poor RNA amplification or labeling (see Examples). The correlation coefficients of the murine and bacterial expression profiles from each individual murine pouch versus all other murine pouches indicates the highest correlations were observed within a given time point, and that consecutive time points are also highly related (FIG. 1 and 2). These findings are also observed for the concordance correlations between the medians of each time point for murine expression profiles (FIG. 3A) and *E. coli* expression profiles (FIG. 3B). For the murine microarray, the highest correlations were observed between the consecutive time points 0 hr and 2 hr (0.96), 2 hr and 4 hr (0.96), and 4 hr and 8 hr (0.96). Subsequently, the 8 hr, 12 hr, 20 hr and 24 hr time points are all highly related ($\geq$0.97 for all pairwise combinations), and comprise a distinct group from the earlier time points (FIG. 3A). The 2 hr and 4 hr time points appeared to be transitional, and showed similarities to both the earlier time points as well as the later time points.

For the bacterial expression profiles, the concordance correlation of the medians of the time point samples were determined (FIG. 3B). In general, although similarly homogeneous within a time point, the *E. coli* expression patterns varied more from time point to time point than the murine expression patterns. Although pouch samples from the 0 hr time points differed significantly from all other infected samples, consecutive time points were again more highly related (FIG. 3B). Furthermore, the expression profile of a mid-log phase LB grown culture of *E. coli* was significantly different from all of the infected samples (FIG. 3B). Interestingly, although pouches from the 0 hr, 2 hr, 12 hr, 20 hr and 24 hr time point were homogeneous within each group, the 4 hr and 8 hr samples demonstrated greater heterogeneity (See Examples). This may be due to a larger range in the CFUs (7-9 fold differences) at those time points versus the smaller range (1.8 fold to 3.1 fold) observed for the 0 hr, 2 hr, 12 hr, 20 hr and 24 hr time points, which is reflected in the bacterial growth curve (FIG. 4). One possibility may be greater biological variability at the 4-8 hr inflection point of the growth curve.

Changes in E. coli Gene Expression.

Genes that were differentially expressed by *E. coli* at different time points after inoculation into the murine pouch were selected by controlling the false discovery rate (FDR) of the selected genes (see Examples). Out of the 4125 *E. coli* genes that did not cross-hybridize to murine mRNA, 155 genes showed at least a 1.5-fold change in gene expression *in vivo* with a P-value <=0.05.

Growth.

E. coli CP9 demonstrated rapid growth in the pouch fluid following inoculation, with a doubling time of ~2.8 hours over the first 8 hrs (FIG. 4). The bacteria reached a peak concentration of ~1 x 10^9 CFU/mL within 8 hrs; however, despite the apparent stationary phase kinetics of the growth curve, the mRNA expression profile was not indicative of stationary phase. Genes previously identified to be induced in stationary phase by RpoS were not observed at the 20 hr and 24 hr time points in the infected pouches (*hyaABCDEF*, *otsBA*, *osmY* and *dps*), and stationary phase genes induced independently of RpoS (*rmf*, *rpoS*, *pspABCDE*) were also unchanged in the pouches. Evidence for continuing active bacterial growth was observed 24 hours following inoculation by the induction of genes encoding products of central metabolism and many genes involved in translation (See Table 1). In addition, these data suggest that between 12-24 hrs, the host was eliminating the bacteria at a rate roughly equivalent to the rate of multiplication.

The *E. coli* expression profiles indicate that within the granulomatous pouch, the bacteria were not subject to stresses previously associated with an intravacuolar environment. Microarray analysis of *S. enterica* sv. Typhimurium after macrophage phagocytosis revealed the induction of genes involved in the response to reactive oxygen species (ROS), low pH and DNA damage (Eriksson, S. et al., *Molecular Microbiology* **47**, 103-118 (2003)).

However, in the current study, we did not observe an expression profile typical of a response to reactive oxygen species (Table 1). These differences may be due, in part, to the extracellular location of the majority of bacteria in the pouch. The conditions supporting rapid growth of extracellular *E. coli* were consistent with an

aerobic environment, since *E. coli* was observed to downregulate the expression of the anaerobic genes *narU* and *narZ* and the fermentative gene *adhE* (Table 2). Induction of the cytochrome ubiquinol oxidase genes cydAB, that are controlled by Fnr and ArcA in microaerobic P. A. (Cotter, P. A. et al, *Molecular Microbiology.* **25**, 605-15 (1997)) was also observed (Table 2).

Table 1. Relative changes in gene expression after infection. Values were normalized to the 0 time control. A total of 155 genes were selected as having at least one time point that met the criteria of >=1.5-fold change and an associated p-value of ≤0.05. Values in bold indicate that both criteria were met.

GeneName	2hr	4hr	8hr	12hr	20hr	24hr
AckA	1.44	1.18	1.19	1.21	1.37	**1.59**
AcnA	**0.57**	**0.59**	**0.60**	**0.58**	**0.57**	**0.55**
Acs	**0.49**	**0.53**	**0.54**	**0.51**	**0.51**	**0.53**
adhE	1.03	1.01	0.94	0.64	0.74	**0.65**
aldA	**0.55**	**0.59**	**0.62**	**0.57**	**0.56**	**0.54**
aldB	**0.26**	**0.27**	**0.28**	**0.28**	**0.29**	**0.29**
amyA	0.71	**0.60**	**0.56**	**0.56**	**0.57**	**0.59**
ansB	1.06	1.10	1.24	1.23	**1.60**	**1.51**
argG	**0.61**	**0.63**	**0.60**	**0.58**	**0.55**	**0.56**
argT	0.69	**0.66**	**0.66**	0.69	0.68	0.71
artP	**0.66**	0.71	0.69	**0.66**	**0.65**	**0.66**
artQ	0.68	0.75	0.72	**0.67**	**0.64**	**0.66**
aspA	0.99	1.22	1.42	1.40	**1.88**	1.29
astB	**0.53**	**0.58**	**0.56**	**0.53**	**0.54**	**0.56**
astD	**0.56**	**0.64**	**0.64**	**0.56**	**0.58**	**0.59**
astE	**0.63**	0.68	**0.66**	**0.64**	**0.64**	0.68
atpD	**1.58**	**1.57**	**1.55**	1.27	1.22	1.21
Bfr	0.68	**0.55**	**0.55**	0.75	0.75	0.71
Cfa	0.72	**0.66**	0.67	0.76	0.78	0.77
cheA	0.87	0.78	**0.63**	**0.53**	**0.53**	**0.53**
cheB	0.90	0.83	0.76	**0.66**	0.68	0.68
cheR	0.84	0.80	0.73	**0.65**	**0.66**	**0.66**
cheZ	0.98	0.83	**0.65**	**0.61**	**0.60**	**0.62**
cirA	**1.86**	1.43	1.46	1.46	1.45	**1.67**

Crr	0.85	0.89	0.84	0.73	0.79	**0.64**
cspA	**1.97**	**2.36**	**2.51**	**4.23**	**4.30**	**2.50**
cspC	**0.58**	0.69	**0.65**	0.81	0.78	**0.59**
cspG	1.44	1.30	1.22	**1.57**	**1.58**	1.19
cycA	**0.55**	**0.53**	**0.51**	**0.52**	**0.51**	**0.51**
cydA	1.31	**1.52**	**1.71**	**1.89**	**2.33**	**2.21**
cydB	1.44	**1.83**	**2.08**	**1.82**	**2.14**	**2.12**
cyoB	0.67	0.75	**0.64**	**0.66**	**0.60**	**0.60**
dcuA	1.12	1.25	1.46	1.37	**1.82**	**1.54**
deoA	**0.62**	**0.65**	0.68	**0.56**	**0.62**	**0.62**
deoB	0.72	0.77	0.83	**0.65**	0.73	0.70
dksA	**0.66**	**0.65**	**0.64**	0.67	0.72	**0.63**
dnaK	**1.54**	**1.55**	**1.83**	**1.53**	**1.62**	**1.96**
dppA	**0.45**	**0.47**	**0.44**	**0.42**	**0.42**	**0.41**
dps	**0.53**	**0.58**	0.67	**0.62**	0.70	**0.62**
ego	**0.47**	**0.49**	**0.66**	0.71	0.83	1.19
entC	1.26	1.24	1.26	1.48	**1.59**	**1.59**
entE	1.37	1.36	1.47	**1.74**	**1.78**	**1.88**
exbB	1.10	1.08	1.15	1.34	**1.54**	1.33
fabF	0.77	0.77	0.69	0.73	0.76	**0.65**
fadA	**0.63**	**0.64**	**0.63**	**0.65**	**0.66**	**0.63**
fadE	**0.49**	**0.52**	**0.54**	**0.50**	**0.50**	**0.49**
fadL	0.70	**0.63**	**0.62**	**0.65**	**0.64**	0.67
feoB	1.15	1.20	1.42	1.49	**1.94**	**1.86**
fepA	1.28	1.16	1.18	1.48	**1.54**	1.39
flgE	**1.77**	1.41	1.15	1.02	1.02	1.03
flgF	**1.60**	1.32	1.16	1.04	1.03	1.04
flgK	1.17	0.93	0.79	0.68	0.68	**0.66**
flgL	1.00	0.89	0.77	**0.66**	0.67	**0.65**
flhA	**1.56**	1.16	0.99	1.04	1.02	1.02
fliC	0.91	**0.62**	**0.36**	**0.32**	**0.31**	**0.30**
fliF	**1.69**	1.13	0.91	1.02	1.04	1.13
fliG	**1.52**	1.08	0.94	1.02	1.03	1.10
FliI	**1.52**	1.23	1.13	1.00	1.00	1.03
fliM	**1.57**	1.08	0.93	1.03	1.07	1.13
fruA	**0.64**	**0.53**	**0.55**	**0.58**	**0.61**	**0.65**
galF	0.68	**0.62**	**0.61**	0.68	0.69	**0.64**
glcA	0.69	**0.64**	**0.63**	0.69	0.71	0.69

glcB	**0.29**	**0.28**	**0.27**	**0.27**	**0.27**	**0.28**
glcE & glcF	**0.58**	**0.59**	**0.55**	**0.55**	**0.55**	**0.56**
glcG	**0.56**	**0.55**	**0.60**	**0.66**	**0.65**	0.76
gltA	**0.51**	**0.52**	**0.54**	**0.55**	**0.54**	**0.54**
gltL	0.71	**0.66**	**0.66**	0.73	0.73	0.78
glyA	0.76	**0.65**	**0.60**	0.79	0.78	0.77
guaB	**0.55**	**0.54**	**0.56**	**0.63**	**0.60**	**0.59**
hisJ	**0.65**	**0.61**	**0.61**	**0.66**	0.68	0.68
hisP	0.68	**0.63**	**0.63**	0.69	0.68	0.72
infB	1.49	1.38	1.17	**1.51**	1.19	1.01
lpp	0.73	0.86	1.01	**1.95**	**1.92**	1.13
mglB	**0.51**	**0.51**	**0.52**	**0.56**	**0.54**	**0.51**
mntH	1.03	0.92	0.98	1.30	**1.51**	**1.72**
moaE	**0.43**	**0.40**	**0.40**	**0.42**	**0.42**	**0.41**
mopA	**1.68**	**1.59**	**1.77**	**1.55**	**1.81**	**2.02**
narU	**0.62**	**0.63**	**0.64**	**0.61**	**0.63**	**0.61**
narZ	**0.60**	**0.59**	**0.60**	**0.60**	**0.61**	**0.58**
nirB	**1.54**	1.10	1.13	1.04	1.11	1.16
nusA	1.41	1.39	**1.54**	1.37	1.19	1.16
ompW	1.18	1.44	**1.75**	1.42	**1.68**	1.23
oppA	**0.40**	**0.38**	**0.35**	**0.42**	**0.42**	**0.41**
osmY	0.69	**0.65**	0.67	0.72	0.74	0.79
pflB	**1.55**	**1.58**	**1.96**	1.42	**1.76**	**1.55**
phoH	**0.34**	**0.32**	**0.33**	**0.33**	**0.35**	**0.35**
proP	0.68	**0.65**	**0.62**	**0.62**	**0.60**	**0.58**
prpE	0.69	0.79	0.75	**0.64**	**0.67**	0.64
purF	**0.58**	**0.49**	**0.48**	**0.63**	**0.57**	0.73
purN	**0.56**	**0.49**	**0.49**	**0.62**	**0.55**	**0.59**
rho	1.08	1.16	1.30	1.45	**1.50**	1.32
rplA	**1.59**	**1.54**	1.44	1.30	1.27	1.24
rplB	2.48	2.63	2.63	2.67	2.00	2.05
rplC	**2.09**	**2.36**	**2.54**	**2.41**	**2.01**	**2.07**
rplD	**1.94**	**2.11**	**2.18**	**2.18**	**1.86**	**1.77**
rplI	**1.84**	**1.64**	1.44	1.30	1.27	1.09
rplJ	**1.90**	**1.68**	**1.71**	**1.57**	1.49	0.94
rplL	**2.21**	**2.10**	**1.96**	**1.76**	**1.65**	1.43
rplO	**0.61**	**0.60**	0.67	0.70	0.81	1.02
rplP	**3.12**	**2.68**	**2.27**	**1.93**	**1.97**	1.59

rplQ	**1.51**	1.34	1.13	1.32	1.32	1.33
rplR	**1.64**	**1.52**	1.32	**1.55**	1.34	1.14
rplU	1.19	1.26	1.32	1.39	**1.65**	1.28
rplV	**2.09**	**2.23**	**1.97**	**1.83**	**1.52**	1.26
rplW	**1.93**	**2.30**	**1.90**	**2.11**	**1.63**	1.35
rpmB	1.23	1.29	1.20	1.37	**1.51**	1.03
rpmC	**1.63**	1.47	1.43	1.24	1.37	1.30
rpmD	**2.07**	**1.73**	1.37	1.50	1.32	1.09
rpmE2	1.01	1.18	1.35	**1.85**	**1.75**	1.42
rpmJ	**1.70**	**1.54**	1.24	1.42	1.33	1.10
rpoB	**1.51**	1.44	**1.51**	**1.61**	1.29	1.14
rpsB	1.43	1.38	1.24	1.33	**1.51**	1.39
rpsC	**2.79**	**2.57**	**2.26**	**2.12**	**1.92**	**1.53**
rpsE	**1.98**	**1.78**	1.50	**1.86**	1.45	1.41
rpsJ	1.42	**1.60**	**1.64**	**1.54**	1.40	1.26
rpsR	**1.50**	1.35	1.21	1.23	1.13	1.01
rpsS	**2.12**	**2.22**	**1.91**	**1.89**	1.41	1.18
sdhA	**0.61**	**0.63**	**0.61**	**0.63**	**0.60**	**0.60**
sdhB	0.82	0.80	0.73	0.80	0.85	**0.66**
secY	**2.69**	**2.29**	**2.07**	**2.01**	**1.95**	**2.04**
sgaA	0.95	0.94	1.10	1.39	**1.50**	**1.52**
sgaH	1.00	1.19	**1.60**	**1.51**	**1.62**	1.36
sgaT	1.00	1.14	1.29	**1.60**	**1.77**	1.37
sgaU	0.99	1.19	**1.73**	**1.59**	**1.61**	1.31
spr	1.06	1.21	1.22	1.35	**1.51**	1.15
tar	0.67	**0.56**	**0.46**	**0.40**	**0.42**	**0.41**
tdcC	1.01	1.09	1.32	**1.59**	**1.80**	**1.75**
tdcE	**1.51**	1.49	**1.50**	**1.53**	1.16	0.96
tsf	**1.50**	**1.51**	1.18	1.30	1.32	1.22
tsr	0.88	**0.56**	**0.41**	**0.42**	**0.42**	**0.46**
uxuB	0.93	1.07	1.41	**1.86**	**1.82**	**2.05**
yadG	0.70	**0.64**	0.68	0.77	0.84	0.72
yciQ	1.12	1.29	**1.58**	**1.60**	1.50	**1.70**
ydcW	**0.48**	**0.47**	**0.48**	**0.50**	**0.53**	**0.49**
yeaG	**0.41**	**0.44**	**0.44**	**0.44**	**0.44**	**0.41**
yeaH	**0.43**	**0.41**	**0.42**	**0.44**	**0.44**	**0.45**
yebA	1.10	1.43	**1.65**	**1.78**	**1.62**	**1.51**
yfbG	**0.60**	**0.59**	**0.59**	**0.60**	**0.59**	**0.59**

yfcX	**0.63**	**0.62**	**0.63**	**0.65**	0.68	**0.64**
yfiD	**1.56**	**1.72**	**2.04**	**1.79**	**2.01**	**1.69**
ygeX	0.96	0.94	1.08	1.24	**1.56**	1.43
ygeY	0.98	1.00	1.24	1.41	**1.68**	**1.60**
ygfS	**0.51**	**0.52**	**0.64**	0.78	0.84	0.99
yghI	**1.68**	1.47	1.17	1.16	1.15	1.07
yhaM	0.93	1.21	1.41	1.46	**1.51**	**1.77**
yhaO	0.94	1.18	1.28	1.40	1.49	**1.83**
yjcG	**0.33**	**0.32**	**0.32**	**0.33**	**0.33**	**0.33**
yjfR	1.00	1.21	**1.97**	**2.18**	**2.37**	**1.96**
yjiY	**2.14**	**1.71**	**1.76**	**1.55**	**1.89**	**2.58**
yliH	0.98	1.09	1.18	1.38	**1.55**	1.44
yncE	1.13	1.08	1.10	1.46	**1.58**	**1.78**
yodA	1.05	**1.53**	**1.83**	**2.45**	**2.15**	**1.58**
yqjE	0.68	0.75	0.75	**0.64**	**0.65**	**0.62**
znuA	1.12	**1.51**	**1.69**	**1.77**	**1.66**	1.48
znuC	1.14	1.35	**1.55**	**1.50**	1.47	1.30

The bacterial expression profile also indicated that oxygen is not limiting within the pouch, as the genes typically induced during anaerobiasis, including *narGHJI*, which encode nitrate reductase, are not induced, and some anaerobic genes are even repressed, such as *narU* and *narZ* and the fermentative enzyme *adhE* (Table 2). In contrast to these data, the cytochrome ubiquinol oxidase genes *cydAB*, that are controlled by Fnr and ArcA in microaerobic conditions (Cotter, P.A., *et al.*, *Molecular Microbiology* 25, 605-15 (1997)) are induced, and the cytochrome oxidase operon, *cyoABCD*, that is repressed by Fnr under anaerobic conditions (Lynch, A. S., *et al.*, *Escherichia coli and Salmonella: Cellular and Molecular Biology*, F.C. Neidhardt, Ed. (ASM Press, Washington, D.C., 1996) Vol. 1, 1526-1538) is down-regulated. These data indicate that the pouch environment is likely either aerobic or microaerobic.

Table 2. Relative changes in bacterial gene expression after infection. Values were normalized to the 0 time control. Numbers in bold indicate a ≥ 1.5-fold change and an associated P-value of ≤0.05. Light gray shading indicates decreases in expression, dark gray shading indicates increases in expression.

Gene Name	Function/description	Time after infection: 2 hr	4 hr	8 hr	12 hr	20 hr	24 hr
	Central metabolism						
adhE	alcohol/acetaldehyde dehydrogenase	1.03	1.01	0.94	**0.64**	0.74	**0.65**
cydA	cytochrome d ubiquinol oxidase subunit I	1.31	**1.52**	**1.71**	**1.89**	**2.33**	**2.21**
cydB	cytochrome d ubiquinol oxidase subunit II	1.44	**1.83**	**2.08**	**1.82**	**2.14**	**2.12**
cyoB	cytochrome o ubiquinol oxidase subunit I	0.67	0.75	**0.64**	**0.66**	**0.60**	**0.60**
narU	nitrate sensor-transmitter protein	**0.62**	**0.63**	**0.64**	**0.61**	**0.63**	**0.61**
narZ	respiratory nitrate reductase 2 alpha chain	**0.60**	**0.59**	**0.60**	**0.60**	**0.61**	**0.58**
	Starvation and stress responses						
cspA	transcription factor	**1.97**	**2.36**	**2.51**	**4.23**	**4.30**	**2.50**
cspC	CspA homolog, not low temperature inducible	**0.58**	0.69	**0.65**	0.81	0.78	**0.59**
cspG	CspA homolog, low temperature inducible	1.44	1.30	1.22	**1.57**	**1.58**	1.19
dksA	dosage-dependent DnaK suppressor protein	**0.66**	**0.65**	**0.64**	0.67	0.72	**0.63**
dnaK	heat shock protein 70	**1.54**	**1.55**	**1.83**	**1.53**	**1.62**	**1.96**
infB	protein chain initiation factor 2, low temperature inducible	1.49	1.38	1.17	**1.51**	1.19	1.01
mopA	chaperone, GroEL protein	**1.68**	**1.59**	**1.77**	**1.55**	**1.81**	**2.02**
nusA	transcription termination/antitermination, low temperature inducible	1.41	1.39	**1.54**	1.37	1.19	1.16
rpoB	DNA-directed RNA polymerase, beta-subunit	**1.51**	1.44	**1.51**	**1.61**	1.29	1.14
yjiY	Homolog of CstA (starvation induced stress	**2.14**	**1.71**	**1.76**	**1.55**	**1.89**	**2.58**

	response)						
	Transport						
argT	ABC transporter: lysine-arginine-ornithine-binding periplasmic protein precursor	0.69	0.66	0.66	0.69	0.68	0.71
artP	ABC transporter: arginine transport ATP-binding protein	0.66	0.71	0.69	0.66	0.65	0.66
artQ	ABC transporter: arginine transport system permease protein	0.68	0.75	0.72	0.67	0.64	0.66
crr	pts system, glucose-specific IIA component	0.85	0.89	0.84	0.73	0.79	0.64
cycA	d-serine/d-alanine/glycine transporter	0.55	0.53	0.51	0.52	0.51	0.51
dcuA	C4-dicarboxylate transporter	1.12	1.25	1.46	1.37	1.82	1.54
dppA	periplasmic dipeptide transport protein precursor, peptide chemotaxis	0.45	0.47	0.44	0.42	0.42	0.41
ego	putative ABC: transporter ATP-binding protein	0.47	0.49	0.66	0.71	0.83	1.19
fruA	pts system, fructose-specific IIBC component	0.64	0.53	0.55	0.58	0.61	0.65
glcA	glycolate permease	0.69	0.64	0.63	0.69	0.71	0.69
gltL	ABC transporter: glutamate/aspartate transport ATP-binding protein	0.71	0.66	0.66	0.73	0.73	0.78
hisJ	ABC transporter: histidine-binding periplasmic protein	0.65	0.61	0.61	0.66	0.68	0.68
hisP	ABC transporter: histidine transport ATP-binding protein	0.68	0.63	0.63	0.69	0.68	0.72
mglB	d-galactose-binding periplasmic protein precursor, active transport and chemotaxis of galactose and glucose	0.51	0.51	0.52	0.56	0.54	0.51
oppA	ABC transporter: periplasmic oligopeptide-binding protein precursor	0.40	0.38	0.35	0.42	0.42	0.41
proP	proline/betaine transporter	0.68	0.65	0.62	0.62	0.60	0.58

sgaA	L-ascorbate transport pts enzyme IIA-like protein	0.95	0.94	1.10	1.39	1.50	1.52
sgaT	L-ascorbate transport pts enzyme IIC protein	1.00	1.14	1.29	1.60	1.77	1.37
yadG	hypothetical ABC transporter	0.70	0.64	0.68	0.77	0.84	0.72
yjcG	hypothetical transporter in sodium:solute symporter family	0.33	0.32	0.32	0.33	0.33	0.33
	Metal ion binding and acquisition						
bfr	bacterioferritin	0.68	0.55	0.55	0.75	0.75	0.71
cirA	colicin I receptor precursor	1.80	1.43	1.46	1.46	1.45	1.67
dps	ferritin homolog, binds & protects DNA, stress & stationary phase inducible	0.53	0.58	0.67	0.62	0.70	0.62
entC	enterobactin biosynthesis: isochorismate synthase	1.26	1.24	1.26	1.48	1.59	1.59
entE	enterobactin biosynthesis: 2,3-dihydroxybenzoate-AMP ligase	1.37	1.36	1.47	1.74	1.78	1.88
exbB	uptake of enterochelin	1.10	1.08	1.15	1.34	1.54	1.33
feoB	ferrous iron transport protein b	1.15	1.20	1.42	1.49	1.94	1.86
fepA	ferrienterobactin receptor precursor	1.28	1.16	1.18	1.48	1.54	1.39
mntH	H(+)-stimulated selective manganese uptake, tranports other metals as well	1.03	0.92	0.98	1.30	1.51	1.72
ompW	outer membrane protein, acts as a receptor for colicin S4	1.18	1.44	1.75	1.42	1.68	1.23
yebA	hypothetical metalloprotease distal to *znuA* and likely co-regulated	1.10	1.43	1.65	1.78	1.62	1.51
yodA	homolog of *S. pneumoniae* zinc-binding protein AdcA	1.05	1.53	1.83	2.45	2.15	1.58
znuA	ABC transporter: zinc binding periplasmic protein	1.12	1.53	1.69	1.77	1.66	1.48
znuC	ABC transporter: zinc transport ATP-binding protein	1.14	1.35	1.55	1.50	1.47	1.30

Bacterial Stress Response.

We observed significant changes in gene expression for several genes either known or suggested to be involved in a stress response. However, the unique pattern of expression was inconsistent with any single previously described *in vitro* stress response such as to antimicrobial agents, oxidative stress, heat shock or acid shock (Yura, T. *et al.*, in *Bacterial Stress Responses* G. Storz, R. Hengge-Aronis, Eds. (ASM Press, Washington, D.C., 2000) pp. 3-18), (Storz, G.*et al.*, in *Bacterial Stress Responses* G. Storz, R. Hengge-Aronis, Eds. (ASM Press, Washington, D. C., 2000) pp. 47-59), (Foster, J. W. in *Bacterial Stress Responses* G. Storz, R. Hengge-Aronis, Eds. (ASM Press, Washington, D. C., 2000) pp. 99-115), (Shaw, K. J. *et al.*, *J. Mol. Microbiol. Biotechnol.* **5**, 105-122 (2003). As part of the novel combination of stress-induced genes, two heat shock chaperones, *dnaK* and *mopA*, were induced in the infected pouches, yet the expression of the >40 remaining genes comprising the heat shock response were unchanged (See Table 1 and Table 2). The *dnaK* and *mopA* genes represent a subset of the RpoH regulon, whose induction by misfolded proteins has been previously observed (Lesley, S. A. *et al.*, *Protein Engineering.* **15**, 153-60 (2002)). Alterations in the bacterial membrane or in the state of membrane-bound proteins could also result in the induction of the heat shock chaperone genes *dnaK* and *mopA*.

In addition to the two heat shock chaperones, a number of genes within the cold shock stimulon were also suprisingly induced (Table 2). These included *nusA*, *infB*, *cspG* and *cspA*, the latter encoding the major cold shock protein (Gualerzi, C. O. *et al.*, *J. Mol. Biol.* **331**, 527-539 (2003). However the related gene *cspC*, which is not part of the cold shock stimulon, did not respond to the same signal, and was repressed (Table 2). The induction of the cold shock genes *cspA* and *cspG* within the infected pouches was also consistent with perturbations in translational or post-translational processes within the bacteria, since their expression is also induced by misfolded protein (Lesley, S. A. *et al.*, *Protein Engineering.* **15**, 153-60 (2002)). In *Staphylococcus aureus*, CspA appears to be an immunodominant antigen that elicits a humoral immune response during systemic infection (Lorenz, U. *et al.*, *FEMS. Immunol. Med. Microbiol.* **29**, 145-153 (2000). In addition, human neutrophils produce an antimicrobial peptide

derived from cathepsin G, which requires CspA production in *S. aureus* to be effective (Katzif, S.*et al.*, *Infection & Immunity* **71**, 4304-12 (2003).Interestingly, we observed induction of the gene encoding cathepsin G in the murine pouch in response to the bacterial infection (see below).

Other stress response genes were also affected in this infection model including elevation in the expression of *yjiY*, which encodes a homolog of the carbon starvation inducible gene, *cstA*, and reduction in the expression of *dps,* which is involved in DNA protection during starvation. Although the signal for the induction of this unique pattern of stress responses is unclear, the data suggest that in this infection model, alternative signals, such as misfolded proteins or host-derived factors, may be triggering these responses.

Transport and Metal Ion Binding and Acquisition.

During the time course of the infection, a decrease in expression was observed for 18 genes known to be involved or having a putative role in transport (Table 2). On the other hand, many genes involved with uptake and binding of metal ions were induced in the murine pouch in a time dependent fashion.

Microorganisms have an absolute requirement for iron and other metals since they are components of critical enzymes involved in catalysis, structure stabilization, and in the biosynthesis of cellular enzymes and macromolecules. Part of the mammalian innate defense strategy is to limit the availability of free metals via binding to serum proteins such as transferrin, ferritin, lactoferrin and hemoglobin. Thus, the levels of free extracellular iron (~ 10^{-18} M), and zinc (~ 10^{-10} M) are very low (Hante, K., *et al.*, in *Bacterial Stress Responses*, G. S. A. R. Hengge-Aronis, Ed. (ASM Press, Washington, D.C., 2000) 275-288). Bacteria have therefore developed a variety of mechanisms to obtain iron, zinc and other critical metals. We observe induction of 12 genes that are involved in or have a putative role in metal ion acquisition including: the putative zinc transport regulon *znuAC* and *yebA,* the putative zinc-binding lipoprotein *yodA*; *entC* and *entE,* which are involved in the synthesis of enterobactin, a powerful iron-chelating agent; the ferrous iron transport protein *feoB*;

the iron chelating siderophore *febA*, which is used by *E. coli* to extract iron from the environment; the *mntH* gene involved in manganese uptake that can also transport iron, zinc, cadmium and colbalt; and *cirA*, the colicin I receptor precursor which may participate in iron transport.

Although essential, metals may also be toxic, due to the promotion of oxidation and lipid peroxidation. Storage proteins, which contain multiple binding sites for metal ions, serve to detoxify metal ions, while retaining internal supplies for mobilization under restricted conditions. Unlike the genes involved in iron and zinc acquisition, which are derepressed under low metal ion conditions, two genes, which have been suggested to play a role in iron storage and detoxification, were down regulated (Table 2). These genes encode bacterioferritin (*bfr*) and a ferritin homolog (*dps*), which can store iron or bind H_2O_2 (Andrews, S. C., *et al.*, *FEMS Microbiol. Rev.* 27, 215-237 (2003)).

Changes in Murine Gene Expression and protein levels.

Genes that were differentially expressed in the infected murine pouches at different time points after inoculation were selected by controlling the false discovery rate (FDR) of the selected genes (see Examples). Over the 24 hr time course of the infection, 682 unique genes that did not cross-hybridize to *E. coli* mRNA demonstrated a >=1.5-fold change in expression that was statistically significant ($P \leq 0.05$). Most of the changes in gene expression were due to the bacterial infection, since we only observed <20 differentially expressed clones in the control uninfected murine pouches over the time course of the experiment (see Table 5). The 682 genes could be grouped into several functional families including defense and immunity, cytoskeleton rearrangement, intracellular signaling, transport, response to oxidative stress, metabolism and apoptosis. Many of these genes have been previously associated with a response to bacterial infection or lipopolysaccharide (LPS) challenge, validating this model; however, we also observed several novel changes in expression.

Croton oil is an irritant that when applied to the skin directly stimulates the release of cytokines and mediators from cells such as keratinocytes and dendritic cells, resulting in the production of an inflammatory reaction characterized by edema and a neutrophilic inflitrate (Catalina, M. D., *et al.*, *J. Exptl. Med.* 184, 2341-2351 (1996). Injection of croton oil injection into an intradermal air pouch results in the formation of a cavity lined with granulomatous tissue and filled with serosanguineous fluid. Besides neutrophils, other cell types are known to be present in the pouch fluid including lymphocytes, monocytes and basophils (Dalhoff, A., *et al.*, *Infection* 10, 354-360 (1982)). Thus, the gene expression observed in the pouch fluid likely reflects the combined expression of these cell types. The expression of histidine decarboxylase, the enzyme responsible for histamine synthesis, confirms the presence of basophils and mast cell in the pouch (Table 3).

Table 3. Relative changes in murine gene expression. Values were normalized to either the infected time 0 or PBS control time 0. Numbers in bold indicate a $\geq$ 1.5-fold change and an associated P-value of $\leq$0.05. Light gray shading indicates decreases in expression, dark gray shading indicates increases in expression.

		Time after infection						Time after PBS		
Gene Name	Function/Description	Infected 2hr	Infected 4hr	Infected 8hr	Infected 12hr	Infected 20hr	Infected 24hr	Control 4hr	Control 12hr	Control 24hr
	Acute Phase Proteins and Vasoactive Mediators									
c3	complement component C3	1.30	2.17	4.09	5.28	6.07	5.48	0.87	1.10	1.02
hamp	hepcidin antimicrobial peptide	1.11	1.64	2.66	2.89	2.27	2.20	0.90	1.16	1.19
hdc	histidine decarboxylase	4.61	13.03	19.44	17.71	13.54	19.78	0.63	1.30	1.00
hp	haptoglobin	2.57	15.46	18.72	17.53	16.25	18.44	0.84	1.26	0.96
mt1	metallothionein 1	1.24	2.54	1.96	1.42	1.83	1.30	0.99	1.00	1.04
saa4	serum amyloid A4	0.93	**0.53**	**0.46**	**0.37**	**0.37**	**0.41**	0.93	**0.61**	**0.54**

	Chemokines and Cytokines									
ccl3	chemokine (C-C motif) ligand 3; MIP-1α	1.01	0.98	0.98	1.00	1.03	1.03	0.97	0.99	1.01
ccl4	chemokine (C-C motif) ligand 4; MIP-1β	0.99	0.98	0.98	1.02	0.97	1.03	0.92	0.93	0.94
ccl5	RANTES	1.61	1.66	0.57	0.45	0.44	0.38	1.06	0.76	0.69
il1b	interleukin 1β	10.38	11.36	10.04	10.35	11.36	10.47	0.56	1.58	0.91
tnf	tumor necrosis factor α	8.86	6.01	7.56	8.32	7.01	8.91	0.83	0.97	1.18
	Oxidative Stress and Responses									
ctsg	cathepsin G	1.06	1.54	1.52	1.29	1.32	1.21	0.92	1.07	0.89
ggt1	gamma-glutamyl transpeptidase	2.61	6.18	8.59	9.35	7.46	8.13	0.96	1.09	1.06
gstz1	glutathione transferase z1 (maleylacetoacetate isomerase)	2.52	8.56	16.31	21.69	20.34	16.01	1.26	1.49	1.09
nos2	inducible nitric oxide synthase 2	0.94	0.71	0.69	0.63	0.67	0.62	0.84	1.05	1.24
sod2	superoxide dismutase 2, mitochondrial	8.74	10.68	14.13	14.11	11.86	15.31	0.95	1.23	1.09

Initiation and development of an acute phase response (APR).

The acute phase response is the systemic response to local inflammation and involved many organ systems including brain, liver, bone marrow and adrenal glands. The activation of the APR in the liver is initiated by IL-1α, IL-1β, IL-6 and TNFα and results in the induction of acute phase plasma proteins (APPs) such as complement proteins, C-reactive protein, haptoglobin and metallothionen. Many of the APPs are important components in innate immunity that exert critical early protective functions to isolate and destroy causing infections and participate in the subsequent restoration of systemic homeostasis (Baumann, H., *et al.*, *Immunol Today* 15, 74-80 (Feb. 1994)).

A dramatic increase in the protein levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα in the early hours post-bacterial inoculation (FIG. 5A) was observed. Similarly, significant changes in the mRNA levels of IL-1β and TNFα mRNA (Table 3) were also seen. While the liver is typically thought of the primary source of APPs, a significant local increase in the level of RNA of several APPs in the pouch fluid, including complement C3, haptoglobin, metallothionen 1, and hepcidin (Table 3) was seen.

One key component of the APR is the induction of both hypoferremia and hypozincemia in plasma, which are important mechanisms in controlling *in vivo* growth of bacteria by limiting the amounts of free iron and zinc and avoiding free-metal induced cellular damage (Falchuk, K. H., *N Engl J Med* 296, 1129-34 (May 19, 1977); Brown, K. H., *Am J Clin Nutr* 68, 425S-429S (Aug. 1998)). The precise mechanism by which hypoferremia is produced *in vivo* has not been completely elucidated, but likely involves hepcidin. Hepcidin is a small cationic peptide with antimicrobial activity, synthesized predominantly by hepatocytes, up-regulated during the APR, and shown to play an important role in iron homeostasis (Ganz, T., *Isr Med Assoc J* 4, 1043-5 (Nov. 2002); Nicolas, G., *et al.*, *Blood Cells Mol Dis* 29, 327-35 (Nov.-Dec. 2002)). Hepcidin-deficient mice do not exhibit hypoferremia in response to turpentine-induced inflammation, suggesting a critical role for hepcidin in the development of hypoferremia during inflammation (Ganz, T., *Isr Med Assoc J* 4, 1043-5 (Nov. 2002)). A significant ~3 fold increase in the expression of hepcidin mRNA (Table 3) was observed, suggesting that host responses resulting in hypoferremia could be induced locally. Local production of hepcidin could inhibit iron release by macrophages, known to be the major site for iron in the body.

Pathogenic bacteria possess many elegant strategies for obtaining iron and zinc *in vivo*. Bacterial iron acquisition mechanisms include the production of siderophores that chelate free iron with high affinity, and systems that allow the bacteria to utilize heme from mammalian heme-containing proteins, such as hemoglobin, haptoglobin, and hemopexin, in low free-iron containing environments (Genco, C. A., *et al.*, *Mol. Microbiol.* 39, 1-11 (2001)). In particular, bacteria are also known to express high

affinity zinc uptake systems, including *znuACB* (Patzer, S. I., *et al.*, *J. Biol. Chem.* 275, 24321-24332 (2000)). In the present study, we observed a significant induction of bacterial genes involved in zinc and iron acquisition from 8-24 hours post infection (Table 2), when localized hypoferremia and hypozincemia was likely to be present (Table 3, FIG. 5A & B). The coordinated induction of these genes suggest a bacterial response to the free metal challenge posed by the host.

Also observed was a ~2 fold increase in expression of metallothionein 1 from 2-4 hours post infection (Table 3). Metallothioneins are intracellular metal binding proteins that are induced during the APR and by oxidative stress whose expression is correlated with an increased uptake of zinc by the liver, bone marrow and thymus, and a concomitant decrease in plasma zinc levels (Brown, K. H., *Am J Clin Nutr* 68, 425S-429S (Aug. 1998)). The exact role of the increased levels of zinc in the liver and decreased levels of zinc in the plasma is not known, but MT-/- mice treated with LPS display decreased levels of glucose in the liver implying a deficit in hepatic gluconeogenesis. Metallothioneins also act as antioxidants and have been reported to scavenge superoxide anions and hydroxyl radicals.

Oxidant – killing and protection from oxidative stress.

The innate immune response is primarily composed of "professional" phagocytic cells that recognize and respond to the microbial invasion. Activated phagocytes generate oxygen-related free radicals and the released oxygen species play a major role in the bactericidal mechanisms of the engulfed organisms. However, the balance between bacterial and host cytotoxicity must be monitored in order to avoid oxidative injury to the host, and thus controlling the respiratory burst activity is crucial to maintenance of this immunological balance. Changes in expression in several genes previously associated with these oxidative killing and host cytoprotection, such as the considerable induction of gamma-glutamyltransferase (GGT1), which is part of the antioxidant protection system were observed (Table 3). Interestingly, purified human GGT has been shown to paradoxically generate reactive oxygen species (ROS) in the presence of molecular oxygen and iron or copper ions (Drozdz, R., *et al.*, *Free Radic*

Biol Med. 25, 786-92 (1998)). Thus the pro-oxidant versus antioxidant role of GGT in this infection model is unclear. In a study examining the induction of GGT by several recombinant human cytokines, IL-1β and TNFα were shown to significantly increase GGT activity in a dose and time-dependent fashion, with GGT mRNA peaking by 3 to 6 hours (Miller, A. M., *et al.*, *Exp Hematol.* 21, 9-15 (1993)). The data in Table 3 are consistent with these finding, since we observe significant (8-10 fold) induction of TNFα and IL-1β mRNA and protein by 2 hrs (Table 2, FIG. 5), with GTT expression peaking slightly later, at 8hrs (Table 3).

A striking (20-fold) time dependent increase in RNA levels for glutathione transferase zeta 1, a distinctive GST enzyme that exhibits glutathione peroxidase activity with the pro-oxidant model substrates t-butyl and cumene hydroperoxides was observed (Lantum, H. B. M., *et al.*, *Drug Metabolism and Disposition* 30, 616-625 (2002)). A previous study showed that expression of this protein, although most pronounced in liver hepatocytes, was seen in a variety of tissues including testis, the epithelial lining of the gastrointestinal tract and prostatic fluid (Lantum, H. B. M., *et al.*, *Drug Metabolism and Disposition* 30, 616-625 (2002)). It has been proposed that GSTs in the testis provide germ cell protection from the products of oxidative stress (Listowsky, I., *et al.*, *Chem. Biol. Interact.* 111-112, 103-112 (1988). The time course and high level induction of this gene in response to the bacterial infection (Table 3) suggests that this enzyme may be involved in the metabolism of oxygen-related radicals and species released by phagocytes during infection.

Maintaining mitochondrial integrity is of utmost importance for normal cellular function, especially during infection. Oxygen radicals produced at the site of infection to destroy the invading bacteria can also damage critical mitochondrial enzymes, rendering the cell unable to generate energy. To counter this oxidative damage, host cells dramatically (up to 15-fold) up-regulate SOD2, or manganese/mitochondrial superoxide dismutase, which specifically protects mitochondria from oxidative damage (Table 3). The crucial role of SOD2 in preserving mitochondrial function was demonstrated by the generation of SOD2-deficient mice, which die within the first 10 days of life (Huang, T. T., *et al.*, *Ann NY Acad of Science* 893, 95-112 (1999)). We

also observe a dramatic (10-15 fold) induction of manganese superoxide dismutase (Table 3).

Although increased expression of several murine genes that are involved in oxidant/antioxidant responses were observed, one gene that was not upregulated was NOS2. Endothelial cells, monocytes and macrophages up-regulate nitric oxide synthase (NOS2) mRNA in response to a variety of inflammatory stimuli, including LPS, IL-1 and IFN-γ. Nos2-catalyzed nitric oxide production is important in NK cell activation, and has been shown to dramatically inhibit *E. coli* growth in conjunction with either peroxynitrite or hydrogen peroxide production (MacMicking, J., *et al.*, in *Annu. Rev. Immunol.*, W. E. Paul, Ed. (Annual Reviews, Inc., Palo Alto, CA, 1997) Vol. 15, 323-350; Pacelli, R., *et al.*, *J. Experimental. Med.* 182, 1469-1479 (1995)). Given these roles in macrophage and NK cell activation and bacterial killing, we were surprised to note a decrease in NOS2 transcription from 12 to 24 hours post infection (Table 3). While NOS2 transcription has been reported to decrease in a model of LPS-induced pulmonary inflammation 12-24 hours post challenge, this decrease was preceded by a dramatic (>10-fold) induction of NOS2 mRNA, which was not observed in our study (Agorreta, J., *et al.*, *Am. J. Resp. Crit. Care Med.* 168, 287-296 (2003)). Nitric oxide also inhibits neutrophil recruitment, thus NOS2 may be down regulated to maintain neutrophil recruitment in order to counter the ongoing infection.

Chemokines, cytokines and their receptors.

Measurement of various proteins at t=0 in the pouch fluid as compared to plasma confirmed the presence of elevated levels of various inflammatory and anti-inflammatory mediators including IL-2, IL-3, IL-6, IP-10, KC/Gro-α, LIF, MCP-1, MCP-3, MCP-5, MIP-1β, MIP-2, IL-10, IL-11, IL-12p70, IL-17, TIMP-1 and TNF-α (data not shown). This is consistent with inflammation induced by croton oil.

Several chemokines and early inflammatory mediators are produced in response to bacterial infections and play an important role in host defense through the recruitment of leukocytes to the site of infection and the initiation of the APR, as discussed earlier. Many early inflammatory mediators were observed to specifically increase after inoculation of *E. coli* into the pouch. At the protein level, these included

IL-1α, IL-1β, IL-6 and TNF-α (FIG 5A). Accordingly, IL-1β and TNF-α transcripts were also dramatically elevated throughout the 24 hr observation period. Although many chemokines were elevated at time 0 in the granulomatous pouch, some chemokines specifically increased in response to infection. We detected a significant and prolonged increase in the protein levels of several chemokines, including KC/Gro-α, MIP-1α, MIP-1β, RANTES, and MIP-2, suggesting the host's continuous attempt to recruit leukocytes to the infected site (FIG 5B). However, at the mRNA level, no changes in the expression of the chemokines MIP-1α and MIP-1β were observed, and an early increase in RANTES expression by 4 hours was followed by a decrease at 8 hours postinfection. The discrepancy in RANTES, MIP-1α and MIP-1β protein and mRNA expression could be due in part to our sampling methodology. As described above, the granulomatous pouch is lined with many cell types, including macrophage-derived giant cells, fibroblasts, and the lining likely contains other cell types, including mast cells. These lining cells could contribute to the chemokine protein levels in the pouch fluid through secretion into the pouch lumen, but would fail to be harvested for mRNA analysis.

The invention will now be illustrated in more detail by the following examples.

Example 1: Bacterial strains and media.

Escherichia coli CP9 is a blood isolate cultured from a patient at the National Institute of Health. Its characteristics include beta-hemolysis, no known antibiotic resistance, O4/K54/H5 serotype and possession of a 36.2 kb cryptic plasmid (Russo, T. A., *et al.*, *Infection and Immunity* 69, 6209-6216 (2001)). *E.coli* CP9 was grown overnight with shaking (250 rpm) at 37 °C in Miller's LB broth (BD, Franklin Lakes, NY). The culture was then diluted 1:50 into 20 ml of LB broth, and incubated with shaking (250rpm) at 37 °C until reaching an OD_{600} of 0.8. The cells were collected by centrifugation and resuspended in 20 ml of phosphate buffered saline (PBS). The OD_{600} of the suspension was adjusted to 0.085 in PBS for inoculation into the mature murine pouches.

Example 2: Murine pouch model.

The murine granuloma pouch was created using a modification of a previously published method (Russo, T. A., *et al.*, *J. Infect. Dis.* 169, 112-118 (1994)). Female Balb/c mice were anesthetized by isoflurane inhalation. The backs of the necks were shaved and approximately 2 ml of air was injected intradermally between the shoulders of the mice, to create a space. 0.25 ml of 0.5% croton oil-99.5% olive oil was injected into the space. The pouch was allowed to mature for 5 days, resulting in the formation of an encapsulated space containing 0.1-1.0 ml of serosanguineous fluid. The mature pouches were injected with 0.25 ml of the OD_{600}=0.085 CP9 bacterial suspension or 0.25 ml of PBS control. The pouches were harvested immediately following euthanasia at time points from 0-24 hours. Blood was recovered from the animals via cardiac puncture. The blood was added to Microtainer blood collection tubes with EDTA (BD, Franklin Lakes, NY), and following centrifugation, plasma was collected.

For analysis of the pouch samples, the volume of the fluid recovered from each pouch was estimated. To determine CFU, 10 µl of pouch fluid was diluted in PBS and plated on LB agar. A 15 µl aliquot of pouch fluid from each pouch at a particular time point and condition (infected or control) was pooled. Analyte levels in the time course of pooled pouch fluids were analyzed by Rules Based Medicine Inc. (Rules Based Medicine, Inc., Austin, TX) utilizing a multiplexed flow-based system: Mouse MAP™ (Multi-Analyte Profiles) analysis technology. RNA-Wiz (Ambion Inc., Austin, TX) was added to the remaining pouch fluid, the samples were mixed well and stored at –20 °C. The total RNA from the pouch fluid was recovered by extraction from RNA-Wiz per manufacturer's instructions with the modification of replacing the IpOH precipitation step with purification with a RNeasy column (Qiagen, Valencia, CA). Briefly, 0.2 volumes of $CHCl_3$ was added and the aqueous phase was collected following centrifugation. An equal volume of RLT was added followed by ethanol to 35% final concentration. The mixture was applied to the RNeasy column and the washing and elution were according to the manufacturer's instructions.

Example 3: Polyadenylation and cDNA synthesis of pouch total RNA.

E.coli poly (A) polymerase (PAP) (Amersham Pharmacia Biotech, Piscataway, NJ) was used to add poly A tails to the 3'ends of the RNA isolated from the pouches,

according to the manufacturer's instructions. Briefly, 5 μg of total RNA extracted from the pouches was used as a template for the reaction. The reaction mixture contained 50mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 1 mM DTT, 0.05% BSA, 2 mM ATP and 3 units of *E coli* PAP. The reaction was incubated at 37°C for 20 minutes, followed by purification with a RNA Clean-up Kit (Zymo Research, Orange, CA). The RNA was recovered in 20 μl of H_20.

PowerScript reverse transcriptase (Clontech, Palo Alto, CA) was utilized for cDNA synthesis per manufacturer's instructions with the following modifications. To the total RNA, 0.5 μg of oligo dT-T7 primer (5'-TCTAGTACCTGCTTCACTGCATCTAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTTTTTT-3') (SEQ ID NO:1) and 1μl of dNTP (10mM each) were added followed by heating to 75 °C for 5 minutes. The reaction was brought to 42 °C for 5 minutes followed by the addition of 4 μl of 5X First Strand Buffer, 1μl of dNTP mix, 1μl of RNase Block (Stratagene, La Jolla, CA) and 2 μl of DTT (100mM). The reaction was held at 42 °C for 5 minutes, followed by the addition of 1μl (10 units) of PowerScript reverse transcriptase (Clontech, Palo Alto, CA). The reaction was incubated at 42 °C for 2 hours and was then inactivated by heating to 75 °C for 10 minutes.

Second strand synthesis was accomplished by the addition of 91 μl of H_20, 30 μl of 5X Second Strand Buffer (Invitrogen, Carlsbad, CA), 3 μl of dNTP mix, 4 μl of *E. coli* DNA polymerase I (Invitrogen), 1 μl of RNase H (Epicentre, Madison, WI), 1 μl of *E. coli* DNA ligase followed by a 120 minute incubation at 16 °C. At this point, 2 μl of T4 DNA polymerase was added followed by an additional 10 minute incubation at 16 °C, followed by heat inactivation at 75 °C for 10 minutes. The cDNA was purified by first concentrating the reaction of 100 μl in a SpeedVac followed by purification over a Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA). The columns were washed 3 times with 500 μl of H_20 prior to reaction addition. The purified cDNA was concentrated under vacuum to a volume of 12 μl.

Example 4: T7 RNA polymerase reaction.

The purified product of the cDNA synthesis reaction was added to a AmpliScribe T7 Transcription Kit (Epicentre, Madison, WI) per the manufacturer's instructions with the following modifications. A 30 µl reaction was assembled with 3.0 µl of 10X T7 reaction buffer, 2.25 µl each of 100 mM ATP, CTP, GTP, UTP, 3.0 µl of 100uM DTT, 1.5 µl of Ampliscribe T7 enzyme mix and 1.5 ul of T7 RNA polymerase (200 units/µl) (Ambion, Inc., Austin, TX). The reaction was incubated at 42 °C for 18 hours. DNase I (1.5 MBU) was added to each reaction followed by a 15 minute incubation at 37 °C. The amplified RNA (aRNA) was purified following the addition of 200 ng of polyinosine with a RNeasy column (Qiagen) per the manufacturer's suggestion. Probe synthesis, microarray hybridization and quantitation were performed as previously described by Shaw, K. J., *et al.*, *J. Mol. Microbiol. Biotechnol.* 5(2):105-22 (2003).

Example 5: DNA microarray gene representation

DNA microarrays were spotted onto Corning GAPS slides using an Amersham Biosciences Generation III spotter. Each clone was spotted in duplicate on the array, with the exception of certain control clones that were spotted 4 to 12 times. The murine microarray contained 3981 clones that represented 3534 unique genes. Among the 3534 genes, 14 plant genes were served as negative control, while 4 murine and 2 human genes served as positive controls. Thus 3518 unique murine genes were represented in the array. The *E. coli* array contained 4255 clones that represented 4204 unique genes, including, 14 plant and 3 human genes that served as negative controls. Thus, 4187 *E. coli* genes were represented in the microarray. The Cy3-labeled cDNA probe preparation, hybridization and subsequent washes of the arrays were performed as previously described (Luo, L., *et al.*, *Nat Med* 5, 117-122 (1999)). All arrays were scanned in a ScanArray 4000 (Perkin Elmer Life Sciences, Boston, MA). Quantification was performed using Imagene (Biodiscovery, Marina del Rey, CA).

Data Analysis

Normalization and elimination of cross-hybridizing clones

Each RNA sample was hybridized to two microarray slides run in parallel, yielding quadruplicate data points for each sample. For analysis, the intensities from each data set were scaled to the 75th percentile (the 75th percentile value of each data set was set to 100), followed by log2 transformation and a two-step normalization procedure, as previously described (Shaw, K. J., *et al.*, *J. Mol. Microbiol. Biotechnol.* 5(2):105-22 (2003)).

Six probe samples from uninfected control murine pouches were hybridized to the *E. coli* microarray to identify *E. coli* genes that cross-hybridized to murine RNA. A clone was flagged as a cross-hybridization outlier if the median intensity of the six control samples after the first normalization step was greater than 128. Of the 4255 clones on the *E. coli* microarray, 62 *E. coli* genes and all 3 human control clones were flagged as cross-hybridization outliers in this manner. Similarly, probes synthesized from RNA isolated from a LB grown mid-log phase culture of *E. coli* was hybridized to the murine microarray in order to determine the murine clones that cross-hybridized to *E. coli* RNA. A clone was flagged as a cross-hybridization outlier if the intensity of the *E. coli* control sample after the first normalization step was greater than 128, and the median intensity of all of the murine samples was less than 2 fold of the intensity in the *E. coli* control sample. Among the 3981 clones on the murine microarray, 99 murine clones (representing 98 genes) and all 14 plant clones were flagged as cross-hybridization outliers. All cross-hybridizating clones were eliminated from further analysis. However, since there were sometimes multiple clones representing the same gene, only 59 murine genes were eliminated.

Selection of differentially expressed genes

Each of the six time points post infection (2hr, 4hr, 8hr, 12hr, 20hr and 24 hr) was compared with time 0 to identify the genes that were differentially expressed by *E. coli* after inoculation into the murine pouch. Similarly, each of the six time points post infection (2hr, 4hr, 8hr, 12hr, 20hr and 24 hr) was compared with time 0 to identify the murine genes that were differentially expressed in the murine pouch after *E. coli* infection. In addition, each of the 3 time points (4hr, 12hr and 24 hr) was compared with time 0 to identify the murine genes that were differentially expressed in the pouch after the instillation of PBS alone (control). For each comparison, the differentially

expressed genes were selected with a controlled false discovery rate (FDR) by permutation.

The log2 ratio of each clone was calculated as the difference between the treatment group sample mean and the control group sample mean of the log2 transformed, normalized intensities in the original dataset. A t score and associated P-value were assigned for each clone by comparing the log2 ratio and its standard error measured for the treatment and control groups, using Welch's approximate t Test. 1000 permuted datasets were generated by random shuffling of the covariate (labels of the treatment group or control group) in the original dataset. For each permuted dataset, the log2 ratio and P-value were calculated for each clone, as performed for the observed dataset. A series of fold change and P value cutoffs was then applied to the observed and permutated datasets to identify differentially expressed clones in each dataset. FDR was defined, for a particular selection criterion, as the ratio of the average number of "falsely identified" clones in all permutated datasets to the total number of identified clones in the observed dataset. We chose a fold change of 1.5 and P-value of 0.05 as the final selection criteria as the FDR for the twelve comparisons for both the *E. coli* and infected murine pouch was between 0.01 and 0.06, while the FDR of the three comparisons in the uninfected pouch was between 0.37 and 1 (Tables 4 and 5).

Out of the 4125 *E.coli* genes that did not cross-hybridize to murine mRNA, we observed 82, 85, 87, 93, and 81 genes that were differentially expressed at the 2 hr, 4 hr, 8 hr, 20 hr, 24 hr time points as compared to 0 hr. Together these genes constituted a list of 155 unique *E. coli* genes since many genes were selected in multiple time points. Out of the 3882 murine clones that did not cross-hybridize to *E. coli* RNA, we observed 118, 443, 607, 618, 592, and 615 clones that were differentially expressed at the 2 hr, 4 hr, 8 hr, 12 hr, 20 hr, and 24 hr time points as compared to 0 hr, with associated FDR ranging from 0.03 to 0.06 (see Table 5). Together these clones constituted a list of 738 unique clones representing 682 unique murine genes. In contrast, we only observed 11, 19, and 13 differentially expressed clones in the uninfected murine pouches at the 4 hr, 12 hr, and 24 hr time points, with associated FDR ranging from 0.36 to 1 (see Table 5).

Table 4. False Discovery Rate (FDR) of differentially expressed *E. coli* clones after inoculation into the murine pouch, using different selection criteria. The numerator in parenthesis indicates the average number of "falsely selected" clones in 1000 permuted datasets and the denominator in parenthesis is the total number of selected clones in original dataset.

Selection Criteria	Infection 2hr	Infection 4hr	Infection 8hr	Infection 12hr	Infection 20hr	Infection 24hr
PV<0.05	0.1 (197.25 / 2002)	0.07 (187.1 / 2612)	0.07 (161.44 / 2239)	0.12 (186.48 / 1619)	0.16 (196.34 / 1197)	0.18 (204.91 / 1146)
PV<0.01	0.03 (42.43 / 1462)	0.02 (33.55 / 2038)	0.02 (23.83 / 1512)	0.03 (33.63 / 1011)	0.05 (32.82 / 692)	0.07 (42.88 / 601)
Fc>=1.5	0.05 (3.95 / 82)	0.04 (3.42 / 85)	0.04 (3.06 / 87)	0.05 (4.23 / 93)	0.05 (5.11 / 97)	0.07 (6.21 / 89)
Fc>=2	0.01 (0.29 / 23)	0.01 (0.2 / 24)	0.01 (0.14 / 24)	0.02 (0.32 / 21)	0.03 (0.62 / 20)	0.02 (0.45 / 23)
PV<0.05, Fc>=1.5	0.02 (1.9 / 82)	0.02 (1.81 / 85)	0.02 (1.55 / 87)	0.02 (1.8 / 93)	0.03 (2.66 / 96)	0.02 (1.69 / 81)
PV<0.01, Fc>=1.5	0.01 (1.08 / 81)	0.01 (0.55 / 84)	0.01 (0.46 / 85)	0.01 (1.13 / 90)	0.01 (0.69 / 86)	0.01 (0.55 / 61)
PV<0.05, Fc>=2	0.01 (0.28 / 23)	0.01 (0.2 / 24)	0.01 (0.14 / 24)	0.01 (0.27 / 21)	0.02 (0.37 / 20)	0.01 (0.16 / 22)
PV<0.01, Fc>=2	0.01 (0.22 / 23)	0 (0.11 / 24)	0 (0.07 / 24)	0.01 (0.19 / 21)	0.01 (0.12 / 20)	0.01 (0.14 / 20)

5

Table 5. False Discovery Rate (FDR) of differentially expressed murine clones with after *E. coli* infection or PBS addition (control) using different selection criteria. The numerator in parenthesis indicates the average number of "falsely selected" clones in 1000 permuted datasets and the denominator in parenthesis is the total number of selected clones in the original dataset.

Selection Criteria	Infection 2hr	Infection 4hr	Infection 8hr	Infection 12hr	Infection 20hr	Infection 24hr	Control 4hr	Control 12hr	Control 24hr
PV<0.05	0.11 (177.71 / 1621)	0.07 (194.01 / 2631)	0.06 (170.14 / 2852)	0.07 (180.26 / 2536)	0.09 (237.8 / 2555)	0.09 (206.03 / 2324)	0.56 (141.34 / 251)	0.49 (113.42 / 231)	0.34 (109.87 / 327)
PV<0.01	0.03 (31.74 / 1010)	0.02 (35.63 / 2098)	0.01 (29.91 / 2408)	0.02 (33.23 / 1938)	0.02 (31.99 / 1795)	0.02 (32.85 / 1642)	0.57 (29.85 / 52)	0.34 (16.86 / 50)	0.24 (15.06 / 63)
Fc>=1.5	0.09 (11.14 / 121)	0.09 (39.6 / 446)	0.11 (64.81 / 607)	0.13 (84.07 / 623)	0.16 (95 / 600)	0.14 (87.28 / 617)	1.96 (58.76 / 30)	0.61 (52.71 / 86)	0.51 (47.81 / 94)
Fc>=2	0.07 (2.8 / 39)	0.06 (9.56 / 155)	0.05 (15.98 / 328)	0.07 (22.56 / 345)	0.08 (27.72 / 337)	0.08 (22.58 / 298)	2 (6 / 3)	0.53 (5.81 / 11)	0.26 (8.68 / 33)
PV<0.05, Fc>=1.5	0.03 (2.97 / 118)	0.03 (13.38 / 443)	0.03 (16.24 / 607)	0.04 (22.69 / 618)	0.06 (33.92 / 592)	0.04 (27.59 / 615)	1.37 (15.03 / 11)	0.49 (9.26 / 19)	0.36 (4.71 / 13)
PV<0.01, Fc>=1.5	0.01 (1.2 / 113)	0.01 (3.84 / 417)	0.01 (4.22 / 600)	0.01 (5.84 / 604)	0.01 (4.22 / 563)	0.01 (6.66 / 567)	1.89 (3.78 / 2)	0.35 (1.75 / 5)	0.23 (0.91 / 4)
PV<0.05, Fc>=2	0.02 (0.8 / 38)	0.02 (3.32 / 155)	0.02 (5.86 / 328)	0.03 (9.09 / 341)	0.04 (12.23 / 337)	0.04 (10.64 / 298)	NA (2.03 / 0)	0.32 (1.27 / 4)	0.3 (0.6 / 2)
PV<0.01, Fc>=2	0.01 (0.41 / 37)	0.01 (1.13 / 153)	0.01 (1.88 / 324)	0.01 (2.22 / 338)	0 (1.44 / 328)	0.01 (2.44 / 288)	NA (0.58 / 0)	0.29 (0.58 / 2)	0.1 (0.1 / 1)

Although the invention has been described above in reference to illustrative examples and preferred embodiments, its scope is intended to not be limited by the foregoing description, but be defined by the following claims as properly interpreted under principles of patent law.

5